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Flow Cytometric Investigation of *Salinicola halophilus* S28 Physiological Response Provides Solid Evidence for Its Uncommon and High Ability to Face Salt-Stress Conditions

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Abstract: In a previous work, some bacterial strains isolated from the Saline di Tarquinia marine salterns (Viterbo, Italy) showed very unusual growth profiles in relation to temperature and salinity variations when grown in solid media. In particular, *Salinicola halophilus* S28 showed optimal or suboptimal growth in a very wide range of NaCl concentrations, suggesting a great coping ability with salinity variations. These intriguing outcomes did not fit with the general *Salinicola halophilus* description as a moderately halophilic species. Therefore, this study profiles the actual physiological status of S28 cells subjected to different NaCl concentrations to provide evidence for the actual coping ability of strain S28 with broad salinity variations. Flow cytometry was selected as the evaluation method to study the physiological status of bacterial cells subjected to different salinity levels, monitoring the strain response at different growth phases over 72 h. Strain S28 showed maximal growth at 8% NaCl; however, it grew very well with no statistically significant differences at all salinity conditions (4–24% NaCl). Flow cytometric results provided clear evidence of its actual and strong ability to face increasing salinity, revealing a good physiological response up to 24% of NaCl. In addition, strain S28 showed very similar cell physiological status at all salinity levels, as also indicated by the flat growth profile revealed in the range of 4–24% NaCl. This is the first study regarding the physiological response during the growth of halophilic bacteria under different conditions of salinity via flow cytometry. This technique represents an effective tool for the investigation of the physiological status of each cell, even if it is somehow underrated and underused by microbiologists for this purpose.

Keywords: *Salinicola halophilus*; flow cytometry; cell physiological status; salinity stress; marine salterns; Saline di Tarquinia

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1. Introduction

Microorganisms are traditionally divided into three categories according to their salt demand for growth: non-halophiles (not requiring salt for growth), halotolerant (growing either in the presence or absence of salt), and halophiles (requiring salt for growth) [1,2]. Halophiles are further subdivided into three categories: slight–, moderate–, and extreme–halophiles, having their optimal growth approximately in the ranges of 2–5%, 5–20%, and 20–30% of NaCl concentration, respectively [3].

Some minor revisions have been proposed to improve this classification, such as the suggested introduction of a new category, "borderline extreme-halophiles", to indicate microorganisms needing at least 12% of salt for their growth [2].

However, the increased number of studies characterizing the adaptive response of extremophilic microorganisms to environmental parameters evidenced that the classical categories are quite unsatisfying to describe the magnitude of microbial biodiversity, and the actual and diversified strategies to cope with environmental stresses. Thus, new categories have been proposed to better describe microorganisms that could barely be included in the traditional groups, showing very uncommon growth profiles in response to variations in chemical–physical parameters, particularly in unstable environments [4,5]. Nevertheless, for various strains, attribution to a well-defined category is still difficult.

Bacteria living in marine salterns, which are constituted by various linked shallow ponds generating an increasing salinity gradient, are subjected to extremely high environmental stress due to very intense daily and/or seasonal fluctuations of chemical–physical parameters (in particular, high variations in temperature and salinity) [6]. The strong environmental heterogeneity establishes complex biological communities distributed along the gradient [7–9], but at the highest salt concentrations, prokaryotic communities predominate and constitute effective models for studying the microorganism distribution pattern in extreme environments, and their adaptation according to the severe living conditions [7,10–15]. The stressful and frequent shifts in water availability and temperature, as demonstrated for other unstable extreme habitats [16–18], render these areas intriguing prototype models of global changes [19].

Saline di Tarquinia (ST) are marine salterns located at ca. 80 km northwest of Rome in the North Tyrrhenian Sea area. ST consist of a hundred-pond system (establishing an increasing salinity gradient) of ca. 135 ha that became a Nature Reserve in 1980. Despite the resultant changes in the system management and some alterations in site fashioning, ST retained extreme tracts, with rapid and broad variations in environmental parameters (primarily in salinity and water temperature) in most of the ponds [8].

In a previous work focused on bacteria isolated from ST, some strains with very uncommon growth profiles in relation to temperature and salinity variations were found. Some of them showed optimal or sub-optimal growth in a very wide range of NaCl concentrations, with broad adaptation to the sudden and repeated fluctuations of salinity [5] that are typical of these hypersaline environments [8]. Among these strains, an emblematic example is that of *Salinicola halophilus* strain S28.

Salinicola halophilus (*Halomonadaceae* family) has been described as a moderately halophilic, mesophilic, Gram-negative, rod-shaped, and motile bacterium [20]. As for other *Salinicola* species, its presence has been reported in a plethora of saline and hypersaline environments (from marine saltern ponds to the phyllosphere of halophytes) from different geographical areas [5,21–23]. *S. halophilus* growth was demonstrated in the salinity range of 3–25%, with the optimum between 7.5 and 10% [24].

A previous work [5] reported that strain S28 grows well in the salinity range of 40–24% with an apparent optimum at 8% salinity. On the basis of these characteristics, it should be considered a moderate–halophile. However, attribution to this category would have just a theoretical value, since its uncommon flat growth profile appeared to definitely be different from that of typical moderate halophiles, which show an evident growth rate drop beyond the optimum. Conversely, strain S28 growth at the (apparent) optimum was quite similar to that at the other tested salinities. Its growth was sub-optimal from 4 to 24% salinity (with no statistically significant differences).

In the mentioned study, the growth rate was measured in solid media (as the daily increase in colony diameter); thus, strain S28's ability to grow well at the different salinity levels should be confirmed in liquid cultures. Providing detailed information on the actual physiological response of the strain subjected to the various salinity conditions is important to understand its actual coping ability. The physiological status can be effectively

investigated by monitoring the injury degree of each bacterial cell subjected to the stressor using multiparametric flow cytometric analysis [25].

In fact, FC has been used to study the bacterial response and cell viability to various physiological stressors, such as ultrasound, UV light [26–28], high pressure [29–31], the effect of various phenolic compounds [32], metal concentration [33,34], antimicrobial substances [35], and temperature or freezing [36,37]. The effect of salt concentration was investigated on lactobacilli [38], halotolerant vibrios [39], and a whole microbial biofilm from a lab-scale wastewater treatment system [40]. By contrast, FC has never been used to investigate halophilic microorganism physiological response.

The current work aims to deepen knowledge about the physiological response of *Salinicola halophilus* S28 to salinity variations through flow cytometric analyses in order to assess the status of bacterial cells grown in liquid media at different NaCl concentrations. To better emphasize strain S28's peculiar coping capacities and unique ability to grow very well and with the same growth rate within a broad range of salinity, its physiological response was compared to that of *Halomonas halmophila* S19. Strain S19, isolated from the same environment, displayed the classical growth profile of a moderately halophilic bacterium with a well-defined optimum and a marked reduction in growth rate beyond the optimal condition.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

Salinicola halophilus S28 and *Halomonas halmophila* S19 were isolated from salty waters collected from a crystallization pond within the Saline di Tarquinia marine salterns [5].

The strains were maintained in the Culture Collection of Marine Bacteria at the Department of Ecology and Biology of the University of Tuscia (Viterbo, Italy), at 4 °C on agar slants of Plate Count Agar (DIFCO) containing 8% Marine Salt (DIFCO), and routinely subcultured.

2.2. Liquid Cultures

Strains S28 and S19 were grown on Luria-Bertani (LB) agar plates containing 8% of NaCl at 30 and 25 °C, respectively (according to their optimal conditions reported by Barghini et al. [5]). After 24 h of incubation, cells were harvested and suspended (10^8 cell/mL) in 8% NaCl solution in distilled water. From this concentrated suspension, the required cell amount to reach a final concentration of 10^6 cell/mL was taken and resuspended in salt-free LB added with different amounts of NaCl (range, 0–28% NaCl; step, 4%). Three replicates for each culture condition (250 mL Erlenmeyer flasks filled with 50 mL of the medium) were incubated in an orbital shaker (180 rpm and 30 or 25 °C for S28 and S19 strains, respectively) for 72 h. Samples taken after 0, 1, 2, 4, 8, 12, 24, 48, and 72 h of incubation were used for the spectrophotometric determination of cell growth (OD_{600}) and the flow-cytometric characterization of the cell physiological status.

All media and NaCl solutions were autoclaved at 121 °C for 20 min.

2.3. Flow Cytometric Analysis

FC analysis was performed at the Center for Scientific Instrumentation (University of Granada, Granada, Spain) using a FACSCanto II cytometer (Becton Dickinson, San José, CA, USA). The flow cytometer was equipped with three laser sets (405, 488, and 625 nm) and detectors for forward–scatter and side–scatter, and eight fluorescence colors. Acquisition from the FC and data analysis (including the calculation of cell subpopulation percentage) was performed by FACSDiva v. 6.1.3 software (Becton Dickinson, USA).

Samples were stained by adding propidium iodide (PI) and 3,3-dihexylocarbocyanine iodide (DiOC6) or fluorescein diacetate (FDA) to reach a final concentration of 1.0, 0.005, and 2.0 µg/mL, respectively. After 15 min of incubation at 30 °C (S28) or 25 °C (S19), stained samples were loaded onto the multiparameter flow cytometer and analyzed.

Different combinations of fluorogenic dyes allowed for characterizing the physiological status of each cell as follows. PI/DiOC6 staining permits revealing the presence of both intact polarized cytoplasmic membranes and active transport systems, which are essential for a fully functional cell. DiOC6 accumulates intracellularly when membranes are polarized or hyperpolarized, indicating a physiological status of metabolically active cells. PI binds to DNA, crossing injured cytoplasmic membranes indicating dying or dead cells; dead cells are red fluorescent [41,42]. In addition, cell viability was tested using the combination of PI and FDA. FDA is a non-fluorescent precursor that is actively transported into viable cells (with an intact cell membrane) and is converted by cellular esterases into a polar membrane-impermeant fluorescent product (fluorescein). This is indicative of cell viability, since cell staining capability requires both effective enzymatic activity (needed for fluorescein production) and cell-membrane integrity (needed for fluorescein retention). Cells having good homeostasis and efficient esterase activity are green fluorescent [42,43].

2.4. Statistical Data Analysis

For the two strains, the growth differences among the various salinity conditions were assessed by non-parametric tests (Kruskal–Wallis, one-way analysis of variance, and Kolmogorov–Smirnov two-sample tests). The statistical analysis of the FC data was performed by parametric tests (one-way analysis of variance and Tukey's test). These tests were performed on the basis of data assessments obtained by the Shapiro–Wilk and Levene tests (performed to test the normal data distribution and homogeneity of variance assumptions, respectively). All the tests were run using the statistical software Systat 8.0 (Systat Software Inc., Point Richmond, CA, USA).

3. Results and Discussion

3.1. Growth in Liquid Medium at Different NaCl Concentrations

In a previous work, *Salinicola halophilus* S28 was reported for its uncommon flat growth curve in relation to different salt concentrations (within 4–24% of salinity) [5].

In the current study, the physiological response of S28 was assessed by FC analysis to provide information on its actual physiological status during growth when submitted to different NaCl concentrations. As a comparison, the same analysis was carried out on *H. halmophila* S19, which showed the classical growth profile with respect to salinity variations characterized by an evident growth rate drop beyond the optimum.

For both strains, the maximal growth was recorded at 24 h of incubation. Figures 1 and 2 report the growth profiles of strains S28 and S19, respectively, in liquid cultures at different salinity conditions (0–28% NaCl concentration, steps of 4%); for each salinity level, the correspondent output of the FC analyses (PI/FDA staining) at different growth times (0, 4, 24, 48 and 72 h) is also shown.

S. halophilus S28 grew well in the range of 4–24% NaCl, with no statistically significant differences among all the salinity conditions ($p = 0.150$, Kruskal–Wallis one-way analysis of variance); maximal growth was observed at 8% NaCl (Figure 1). These results confirmed the previous observations indicating its very uncommon growth profile and provided clear evidence that all salinity conditions (from 4% up to 24% of NaCl) supported the same high growth-rate levels.

A very different growth profile was obtained for *H. halmophila* S19 that had the same salinity growth range (4–24% NaCl), but showed a marked growth decrease at salinities higher than the optimum (4% NaCl) (Figure 2). The optimal condition recorded in this study was a lower salinity level than that of the previous investigation (8% salinity). This would allow for the classification of S19 as a slight rather than a moderate halophile. However, the discrepancy between the optimal values may have been due to the different methods used for growth determination. Growth detection in liquid cultures is reasonably more accurate than that in solid media (growth measured as colony diameter increases).

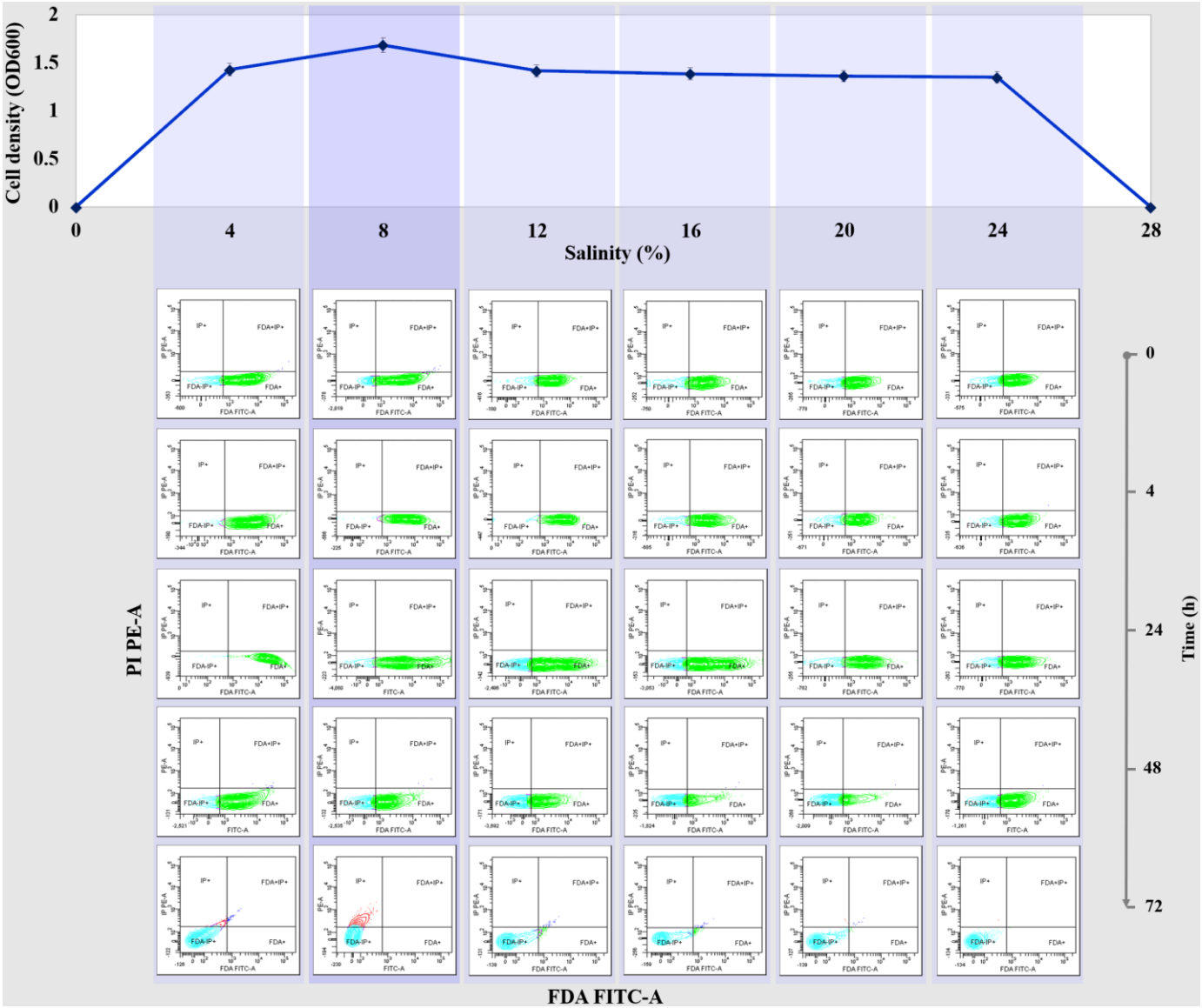

Figure 1. Growth profile and physiological response (FC analysis based on PI/FDA staining) of *Salinicola halophilus* S28 grown in LB containing different concentrations of NaCl. Growth data (OD_{600}) are the mean of three replicates, and standard deviation (bars) was less than 10%. No significant differences in growth among the NaCl conditions were observed (Kruskal–Wallis one-way analysis of variance: $p = 0.150$). Cell subpopulations individuated by PI/FDA double staining: cyan = FDA-/PI-, cells with an intact membrane but absence of esterase activity; green = FDA+/PI-, cells with intact membrane and presence of esterase activity; blue = FDA+/PI+, cells with minimally damaged membrane and presence of esterase activity; red = FDA-/PI+, cells with compromised membrane and absence of esterase activity (dead cells).

The findings of the present work appear somehow not consistent with the literature, which generally describes the species as moderately halophilic [20,44]. However, due to the extremely huge bacterial diversity, it is possible that strain S19 may have different adaptation features from those of other members of the species, driven by the specific ecosystem.

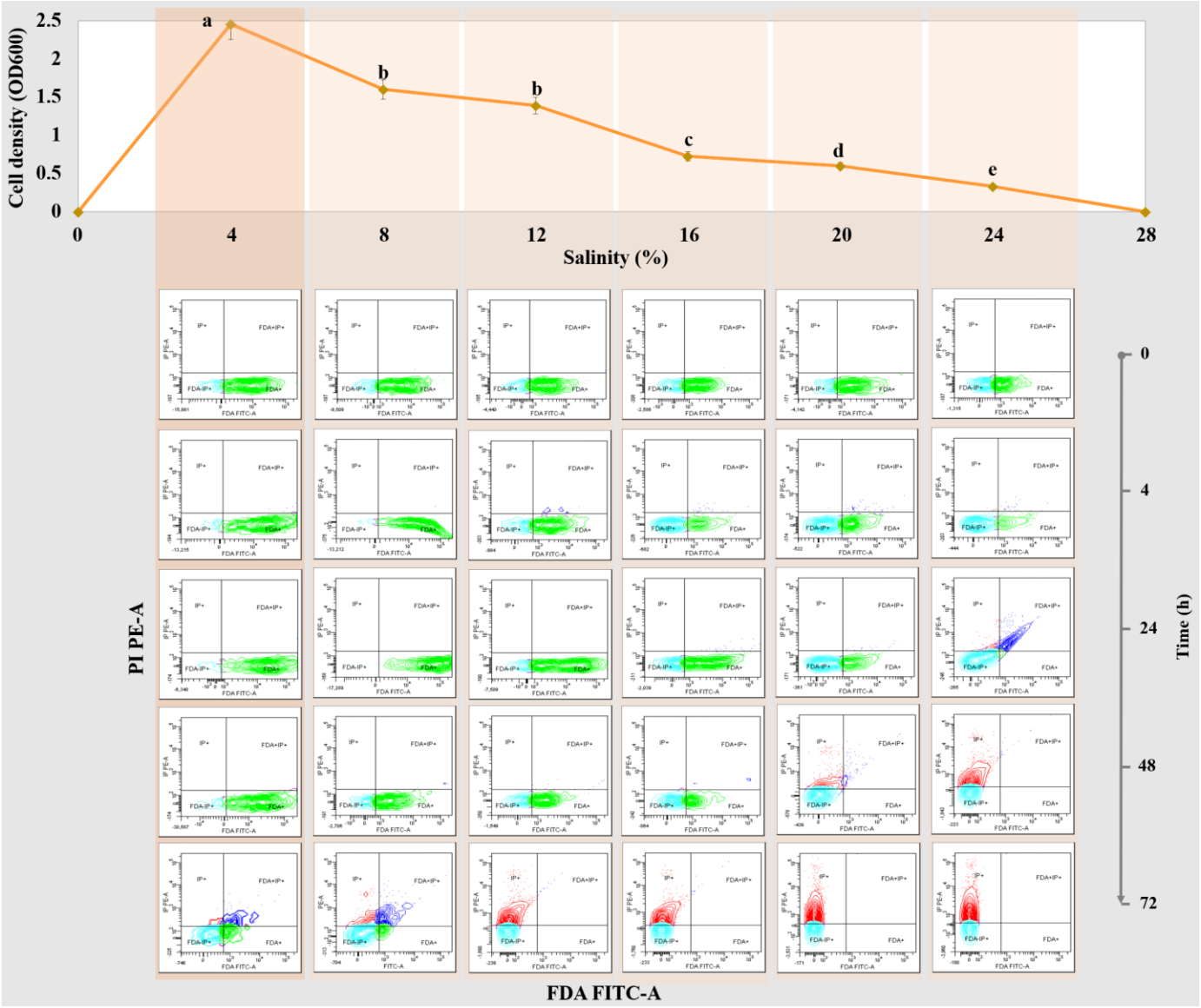

Figure 2. Growth profile and physiological response (FC analysis based on PI/FDA staining) of *Halomonas halmophila* S19 grown in LB containing different concentrations of NaCl. Growth data (OD_{600}) are the mean of three replicates, standard deviation (bars) was less than 10%. The same superscript letters indicate no significant differences in growth between NaCl conditions (Kruskal–Wallis one-way analysis of variance, and Kolmogorov–Smirnov two-sample tests). Cell subpopulations individuated by PI/FDA double staining: cyan = FDA-/PI-, cells with an intact membrane but absence of esterase activity; green = FDA+/PI-, cells with intact membrane and presence of esterase activity; blue = FDA+/PI+, cells with minimally damaged membrane and presence of esterase activity; red = FDA-/PI+, cells with compromised membrane and absence of esterase activity (dead cells).

3.2. Physiological Response at Increasing NaCl Concentrations

FC analysis based on PI/FDA staining allowed for assessing viability and cell damage (evaluating membrane integrity and the presence of intracellular esterase activity) over the incubation time in relation to the different NaCl concentrations. The PI/FDA double staining enabled the individuation of four subpopulations: cells with an intact membrane and the presence of esterase activity (PI-/FDA+), cells with a minimally damaged membrane and the presence of esterase activity (PI+/FDA+), cells with an intact membrane but the absence of esterase activity (PI-/FDA-), and cells with a compromised membrane and the absence of esterase activity (dead cells; PI+/FDA-) [38].

FC profiles clearly demonstrated the great coping ability of strain S28, which had similar physiological responses at all salinities in the range 4–24% NaCl (Figure 1). At these salinities, cells with intact membranes and the presence of esterase activity (PI-/FDA+) represented the highest fraction (45.4–99.7% of S28 total cells) over the first 24 h of incubation. Furthermore, the PI-/FDA+ percentage increased from the early hours to reach the maximum at 24 h, when maximal growth occurred. At 48 h, a decrease in PI-/FDA+ cells and an increase in cells having an intact membrane but not showing intracellular esterase activity (PI-/FDA-) were recorded. At 72 h, almost all cells were PI-/FDA-, and a small fraction (0.1–0.9%) were represented by cells with intracellular esterase activity that began to have a minimally damaged membrane (PI+/FDA+). Unlike the other salinity conditions, more than 25% of the cells were dead (PI+/FDA-) at 72 h of incubation at 8% NaCl (salinity condition supporting maximal growth). Since growth at the best salinity condition is both higher and faster, a fraction of the cells presumably began to die at 72 h due to starvation.

Considering each incubation time, the statistical analysis performed on the abundance (%) of the cell subpopulation at the various salinities showed a certain variability: some abundances were not statistically different ($p < 0.05$; ANOVA followed by Tukey's test), whereas others, although definitely similar, were statistically different. For instance, at 4 h of incubation, the abundances of the PI-/FDA- and PI-/FDA+ subpopulations at 16% salinity were different from those at 20% and 24%; at 48 h of incubation, the abundances of the PI-/FDA- and PI-/FDA+ subpopulations at 16% salinity were different from those at 20%. However, in most cases, these fluctuations may have been due to random factors, and at each incubation time, although some subpopulation percentages between various salinities were statistically different, FC graphs clearly show an overall similar physiological cell status.

The great adaptation ability of S28 (as revealed by its cell physiological status that was very similar at all salinities from 4% up to 24% NaCl) was more evident when compared with that of S19, which showed worse ability to cope with the rising NaCl concentrations (Figure 2). Also for S19, an increase in PI-/FDA+ percentage was observed starting from the early hours to reach the maximum at 24 h (in correspondence with maximal growth), but only at the salinities in the range 4–16% NaCl. At salinities higher than 16% NaCl, an increase in cells having an intact membrane but not showing intracellular esterase activity, and the presence of cells with intracellular esterase activity but a minimally damaged membrane were recorded already after 4 h of incubation. In addition, a rather high presence of injured cells (with slightly damaged or compromised membrane) was observed at 24 h of incubation at 24% salinity; PI+/FDA+ and PI+/FDA- subpopulations represented 15.6% and 1.1% of the S19 cells, respectively. At 48 h, an increase in the cells with intact membrane and no intracellular esterase activity were recorded at salinities in the range of 4–160%, whereas a high presence of dead cells was observed at 20% and 24% NaCl (31% and 84.4%, respectively). At 72 h, the percentage of cells with slightly damaged or compromised membranes increased at all salinities; in particular, a high fraction (60.5–77.8%) of dead cells (PI+/FDA-) was recorded at 12–24% NaCl.

To obtain further insights into the differences in the physiological responses between the two strains, a different combination of fluorogenic staining (DiOC6/PI) was used. Figure 3 shows the comparison of the time evolution of the two strain cell subpopulations at the salinity related to maximal and/or optimal growth (8% NaCl for S28 and 4% NaCl for S19), intermediate salinity (16% NaCl), and at the upper range limit (24% NaCl).

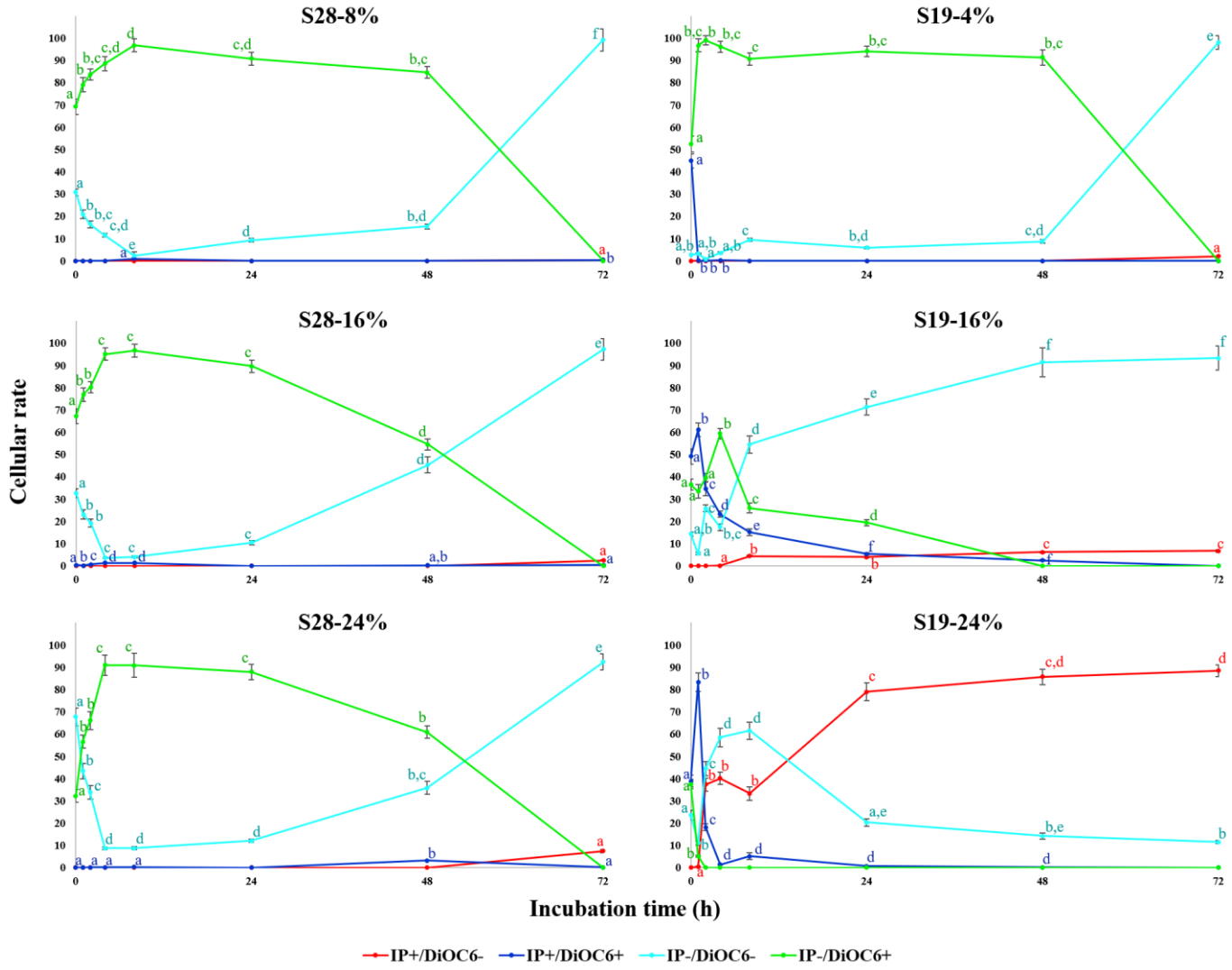

Figure 3. Time evolution of the cell subpopulations of *Salinicola halophilus* S28 and *Halomonas halmophila* S19 grown in LB containing different NaCl concentrations as revealed by FC analysis (DiOC6/PI staining). Time evolution of cell subpopulations was reported for the best salinity condition for growth (8% NaCl for S28 and 4% NaCl for S19), intermediate salinity (16% NaCl), and the upper range limit (24% NaCl). Line colors represent the cell subpopulations individuated by DiOC6/PI staining: cyan = DiOC6-/PI-, cells with an intact and low-polarized membrane (cells in latency); green = DiOC6+/PI-, cells with an intact and highly polarized membrane (metabolically active cells); blue = DiOC6+/PI+, cells with a polarized but permeabilized membrane (suffering cells starting to lose membrane polarization and to acquire PI); red = DiOC6-/PI+, cells with a compromised and depolarized membrane (dead cells). Same superscript letters on colored lines indicate not statistical differences (ANOVA followed by Tukey test; $p > 0.05$) among subpopulation abundance along time.

FC analysis based on DiOC6/PI staining permitted the evaluation of cell physiological status based on membrane integrity and potential. The staining allowed for recognizing the following subpopulations: cells with an intact and low-polarized membrane (cells in latency; DiOC6-/PI-), cells with an intact and highly polarized membrane (metabolically active cells, DiOC6+/PI-), cells with a polarized but permeabilized membrane (suffering cells starting to lose membrane polarization and to acquire PI; DiOC6+/PI+), and cells with a compromised and depolarized membrane (dead cells; DiOC6-/PI+) [32,42].

S28 subpopulation trends at 16% and 24% NaCl were quite similar to those at 8% (maximal growth salinity condition) (Figure 3). Overall, latent and metabolically active cells exhibited specular trends over the 72 h. At 8% NaCl, after 8 h, almost all cells were metabolically active (DiOC6+/IP-). A slight decrease in this subpopulation occurred between 8 and 48 h, with a concomitant slight increase in latent cells (DiOC6-/IP-). A sharp drop in metabolically active cells and a sharp increase in latent cells were recorded between 48 and 72 h of incubation. At 16% and 24% NaCl, after 4 h, almost all cells were metabolically active. A slight decrease in this subpopulation occurred between 8 and 24 h, with a concurrent slight increase in latent cells. The drop in metabolically active cells and the increase in latent cells were more pronounced between 24 and 48 h, and became sharp between 48 and 72 h. These results confirmed those obtained via the combination of PI/FDA staining, stating that S28 could cope well with the salinity increase up to 24% NaCl.

By contrast, S19 showed scarce ability to face increasing NaCl concentrations. At the optimal salinity (4% NaCl), at 2 h of incubation, 99% of S19 cells were metabolically active. A slight decrease in this subpopulation was recorded between 2 and 8 h with a concomitant slight increase in latent cells (DiOC6-/IP-); between 8 and 48 h of incubation, the S19 subpopulation percentage remained almost unchanged. A marked decrease in metabolically active cells and a parallel marked increase in latent cells were observed between 48 and 72 h; in addition, 2% of cells were dead (DiOC6-/PI+) at 72 h. At the optimal salinity, the subpopulation trends of the two strains were quite similar. Compared to S28, S19 showed a very different physiological response at the intermediate and upper limit salinities of the growth range. At 16% NaCl, already within the first hours of incubation, certain strain suffering was recorded, with a rather low percentage of metabolically active cells. At 8 h, the metabolically active cell percentages decreased, while a strong increase in the latent cell fraction was recorded. Between 8 and 48 h, a further increase in latent cells with a concomitant decrease in metabolically active cells were recorded (at 48 h, 91.4% of cells were in latency). Between 48 and 72 h, the percentage of these subpopulations remained almost unchanged. In addition, at 16% NaCl, starting from 8 h of incubation, a percentage of dead cells (4.4%) was recorded, which slightly increased thereafter (up to 6.7% at 72 h). At 24% NaCl, already after 4 h of incubation, 58.5% of cells were in latency, and 40.1% were dead. Between 8 and 24 h, a sharp increase in dead cells was observed (from 33.3% to 79%); at 24 h, 20.3% of cells were in latency. Between 24 and 72 h, the dead cells further increased (up to 88.5% at 72 h), while the latent cells decreased (to 11.5% at 72 h).

4. Conclusions

The current work, representing the first manuscript studying the physiology of halophilic microorganisms by flow cytometry, confirmed the great coping ability of *Salinicola halophilus* S28 with respect to salinity variations suggested by preliminary studies. Although the species is generally considered a moderate halophile, strain S28, due to its unique growth profile, does not fit with the characteristics of this category of halophiles. Its very flat growth profile in a broad range of NaCl concentrations (with no significant growth differences in the range of 4–24% NaCl) has never been reported for other strains of this species and other bacteria. FC results clearly demonstrated the strong ability of S28 to face increasing salt concentrations, as indicated by the good physiological status of the bacterial cells up to 24% salinity. This unusual response was further highlighted by the comparison with that of *Halomonas halmophila* S19, which was able to grow within the same salinity range, but showed a typical marked growth rate drop beyond the optimum.

This work represents a valid starting point for the further detailed study of this strain, which should be oriented towards the understanding of the molecular mechanisms at the core of its uncommon coping ability to broad salinity variations. The study of its genome would also allow for individuating specific traits to test for possible applications. In fact, the strain coping ability could be used in industrial and/or environmental biotechnology, such as the production of salt-tolerant enzymes and the biodegradation of pollutants (as shown by other strains of the species) in halophilic conditions.

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